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Patents

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In re application of

Eva GRAHN HAKANSSON et al. Conf. No. 4905

Serial No. 09/623,562 Group 1651

Filed November 16, 2000 Examiner I. Marx

Title A NOVEL BACTERIAL STRAIN AND USES THEREOF

DECLARATION OF MARIA ISAKSSON UNDER 37 C.F.R. §1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Maria Isaksson, a Swedish citizen residing at Överboda 202, 905 88 Umeå, Sweden, hereby declare as follows:

1. I received my Masters of Science degree in Biomedicine in 2001. Since 2001 I have been working for Essum AB of Umeå, Sweden, as a project coordinator and leader. I have been working in the field of the present investigation utilizing the methods described herein since 2000. I also attended a basic course in clinical trials at the Swedish Academy of Pharmaceutical Sciences in 2002.

2. I obtained two strains of microorganisms and performed tests to compare their relative abilities to inhibit the growth of and to kill common pathogenic urogenital bacteria. The strains evaluated were:

Lactobacillus plantarum 931
Deposit Number DSM11918
Described in U.S. Patent Application No. 09/623,562
Strain obtained from

Deutsche Sammlung von Microorganismen und Zellkulturen
Mascheroder Weg 1b
D-38124 Brauschweg, Germany

and

Lactobacillus plantarum RC-20
Deposit Number ATCC 55883
Described in U.S. Patent No. 5,645,830
Strain obtained from

American Type Culture Collection
PO BOX 1549
Manassas, Virginia 20108 USA

4. Purity of the *Lactobacillus plantarum* RC-20 ("ATCC 55883") was checked upon receipt. The strain was cultured in MRS broth, on blood agar plates (5% horse blood in Columbia agar), and on MRS (Merck) agar plates. Only one type of colony was seen in each culture so the strain was considered pure.

5. The ATCC 55883 was then typed using API 50 CH (bioMérieux, Marcy l'Etoile, France). The result clearly indicated that the strain was not a *Lactobacillus plantarum*, but instead a *Lactobacillus crispatus*.

6. The ability of PBS supernatant from LB931 and the *Lactobacillus crispatus* ATCC 55883 to inhibit or kill pathogens was studied by first preparing the necessary materials.

ATCC 55883 and LB931 were grown individually on MRS agar plates for two days at +37°C in 5% CO_2. One colony of ATCC 55883 and one colony of LB931 were separately inoculated into 5 ml of MRS broth ('start tube') and incubated at +37°C in 5% CO_2 overnight. Then 50 ul from each start tube was separately put into 5 ml of preheated MRS broth and incubated for 8 hours. After incubation, 1 ml was transferred into 100 ml of preheated MRS broth and further incubated for 16 hours.

The suspensions were centrifuged and re-suspended in 10 ml of PBS in a 50 ml Falcon tube. The tubes were incubated at +37°C for 5 hours. Thereafter the suspensions were centrifuged and the supernatants ('PBS sup') collected, containing inhibiting substances secreted by the lactic acid bacteria. The PBS sup was sterile filtered through 45 um and 22 um filters arranged consecutively. The filtered PBS sup fractions were stored at -20°C until use. The pH for the LB931 sup was measured to 3,7 and for the ATCC 55883 sup to 3,9. For controls, pH adjusted PBS (pH 4.1) was used.

7. Pathogens were prepared as well for use in the comparison experiments. Four common urogenital pathogens were studied: Group B *Streptococcus* (GBS, 090), *Enterococcus faecalis* (270), *Staphylococcus aureus* (02-1), and *Escherichia coli* (268). Each pathogen was grown in TH broth and diluted 1:100. Following dilution, 10 ul of each pathogen suspension was inoculated into separate 1 ml fractions of each PBS sup, including control. Into each tube, 5% TH broth was added to promote pathogen growth in the nutriment-poor environment of PBS. Samples were collected at 0, 4, 6 and 25 hours after incubation at +37°C.

8. After incubation with pathogens, the PBS sup of both LB931 and ATCC were observed to inhibit bacterial growth relative to the control. Significant differences in the degree of inhibition and the ability to kill bacteria were observed, however.

9. Regarding GBS, Figure 1 depicts the growth rates measured during the experiment. The PBS sup of ATCC 55883 inhibited growth of GBS as compared to the control. The pathogen was not killed by ATCC 55883, even after 25 hours.

In the PBS sup of LB931, growth of GBS was immediately inhibited and the pathogen was killed after 4 hours.

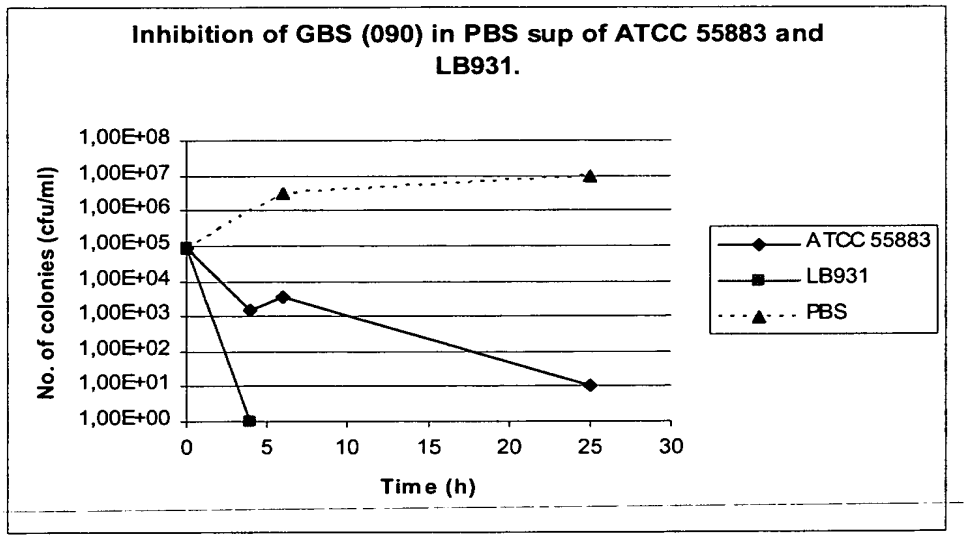

Fig 1. Group B *Streptococcus*

10. Regarding *Enterococcus faecalis*, Figure 2 depicts the growth rates measured during the experiment. The PBS sup of ATCC 55883 inhibited growth of *Enterococcus faecalis* slightly as compared to the control. The pathogen was not killed by ATCC 55883, even after 25 hours.

In the PBS sup of LB931, growth of *Enterococcus faecalis* was immediately inhibited and the pathogen was killed after 6 hours.

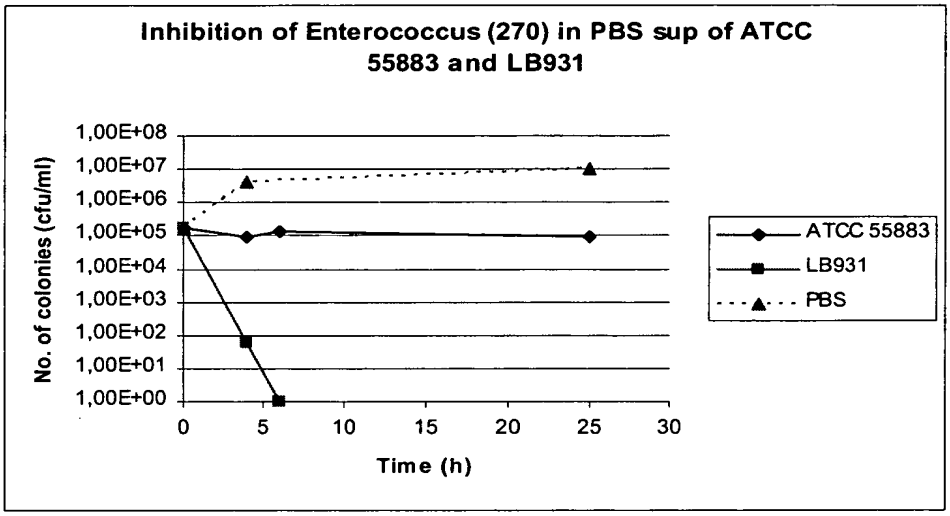

Fig 2. *Enterococcus faecalis*

11. Regarding *S. aureus*, Figure 3 depicts the growth rates measured during the experiment. The PBS sup of ATCC 55883 inhibited growth of *S. aureus* as compared to the control. However, the pathogen was not killed by ATCC 55883, even after 25 hours.

In the PBS sup of LB931, growth of the *S. aureus* was immediately inhibited and the pathogen was killed after 25 hours.

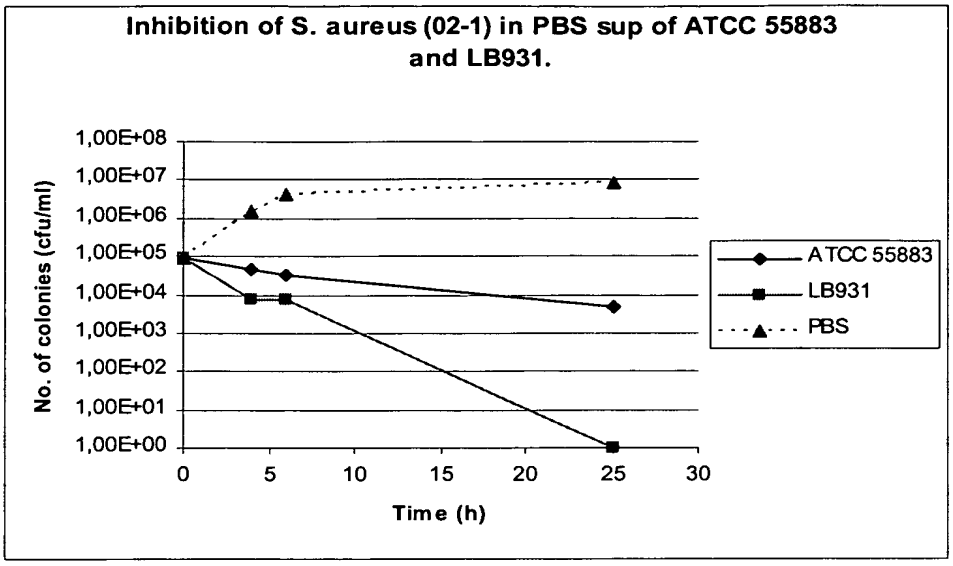

Fig 3. *Staphylococcus aureus*

12. Regarding *Echerichia coli*, Figure 4 depicts the growth rates measured during the experiment. The PBS sup of ATCC 55883 inhibited growth of *E. coli* as compared to the control. The pathogen was not killed by ATCC 55883, even after 25 hours.

In the PBS sup of LB931, growth of *E. coli* was inhibited in the first 6 hours and killed after 25 hours.

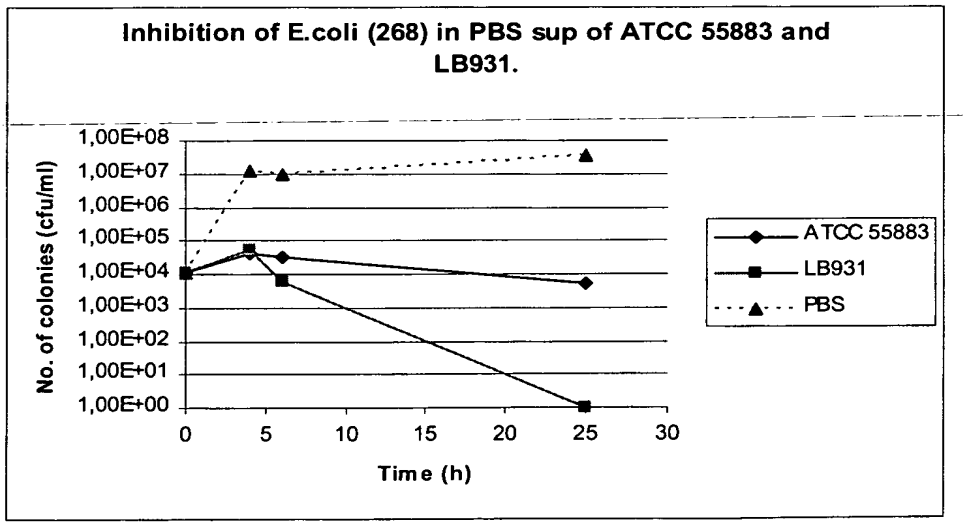

Fig 4. *Echerichia coli*

13. The significance of the above-described results is that there is a pronounced difference in the ability of the strains to inhibit and kill common urogenital pathogens. The LB931 immediately inhibited all pathogens and killed all pathogens by 25 hours after inoculation. The GBS was killed after only 4 hours and the *Enterococcus* after only 6 hours by LB931.

While ATCC 55883 showed some inhibition, occasionally only weak inhibition, even after 25 hours none of the pathogens were totally killed by ATCC 55883. This demonstrates that LB931 shows strong inhibiting and killing effect on four common urogenital pathogens, whereas ATCC 55883 shows only mild inhibiting effect and no killing effect on the same pathogens, when evaluated under the same conditions. Thus, LB931 offers advantages over ATCC 55883 as a probiotic bacterium.

14. I declare that the preceding statements which are made of my own knowledge are true and that the preceding statements which are made on information and belief are believed to be true. I am aware that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Umeå, 8th of December 2003.

Maria Isaksson